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TITLE: Early Host Responses to Prion Infection and Development of an *In Vitro* Bioassay

PRINCIPAL INVESTIGATOR: George A. Carlson, Ph.D.
Leroy E. Hood, M.D., Ph.D.

CONTRACTING ORGANIZATION: McLaughlin Research Institute
Great Falls MT 59405

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George A. Carlson
Leroy E. Hood, M.D., Ph.D.

Email: gac@po.mri.montana.edu

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**14. ABSTRACT**

The goal of this project is to identify pathways and networks of genes and proteins perturbed by prion replication. The unusual nature of prion disease prompted a systems approach to identify networks specifically perturbed by prion infections and to determine which perturbations are essential for various aspects of the disease. We previously tracked changes in gene expression in brain and spleen for two different prion strains and five different lines of mice over their entire incubation periods. We have successfully infected CNS stem cell containing neurosphere cultures with the Rocky Mountain Laboratory (RML) prion strain. Neurosphere lines have been produced from the same mouse strains used for our in vivo studies. Differential gene and protein expression in these cells will aid in identifying genes directly involved in prion replication and lead to the identification of markers for prion infected individuals. We also are developing neurosphere cultures as a sensitive, rapid bioassay for mouse, bovine, and human prions.

**15. SUBJECT TERMS**
Prion, diagnostic, mice, RNA, gene expression, brain, spleen, blood, cell culture

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INTRODUCTION

A misfolded form of prion protein ($PrP^{Sc}$) is the functional component of infectious prions and is derived by posttranslational templating of the normal $PrP^{C}$ isoform (Prusiner, 1998). A hallmark of prion disease is its long incubation period, which is the interval between inoculation and onset of clinical signs of neurological dysfunction. In rodents, the earliest detectable indicator of prion infection is a rise in infectivity. The ability to infect experimental animals is a more sensitive assay for prions than commonly used methods to detect $PrP^{Sc}$ by immunoblotting, which generally requires between $10^5$ and $10^7$ infectious units (IU) per ml. New technologies, such as the conformation dependent immunoassay (Safar et al., 2000; Safar et al., 2005), are providing dramatic increases in sensitivity and further improvements in detection of $PrP^{Sc}$ are likely. However, specific changes in host biology that accompany, or even precede, conversion of $PrP^{C}$ to $PrP^{Sc}$ may provide more sensitive indicators of prion exposure.

Based on the hypothesis that a variety of intracellular and intercellular systems are perturbed at both the protein and gene transcription levels by prion replication, we have applied a discovery-based approach to identify changes induced by prions that can be used as a signature for prion-infected individuals. To identify a gene expression signature specific to prion-infected individuals, we used Affymetrix chips to search for changes in mRNA expression that showed overlap in different mouse strain-prion strain combinations. This work using mice is nearing completion. One impediment to application of arrays to prion disease and to mechanistic analysis of genotype-agent strain interactions has been the lack of a versatile tissue culture assay for prion infection. The ability to infect cells derived from mice of any genotype or from humans would provide a new tool for dissecting prion disease and for validating in vitro assays for infectivity. With support from the Department of Defense National Prion Research Program, we have successfully infected central nervous system (CNS) stem cell (SC) lines from a variety of normal and transgenic mice with mouse scrapie prions. One goal of this project is to develop CNS-SC lines from transgenic mice over-expressing human or bovine PrP that can be used as in vitro bioassays for human and bovine prions. Current bioassays for human or bovine prions require inoculation of transgenic mice and waiting for disease to develop. Although requiring up to two years for definitive results, bioassays in mice are more sensitive than current screening tests based on detection of misfolded, proteinase-resistant PrP. CNS-SC lines also will facilitate our global microarray and proteomics approaches to identify gene expression signatures specific to prion-infected individuals before they are clinically ill. The ultimate goal is to develop sensitive blood tests for humans and animals incubating infectious prions.

BODY

Progress towards completing each specific task in the Statement of Work are indicated in **bold type** below:

*Task 1.* Determine whether there are specific changes in mRNA and protein expression profiles following prion infection in neurosphere (CNS-stem cell) cultures. (Months 1-12) **As noted in the first annual report, preliminary results for two independent neurosphere cultures from Tg(MoPrP-A)4053 infected with the RML strain of prions have been obtained. A subset of the genes differentially expressed in prion infected mice are found in infected neurospheres compared with those exposed to normal brain homogenate. These preliminary results demonstrate that significant results from this approach are feasible.**

*Task 2.* Compare results from neurosphere cultures to results obtained using prion infected mice from peripheral blood cells, spleens, and brains using the same mouse strain-prion strain combinations. Gene expression signatures potentially specific to prion-infected individuals will be identified. (Months 3-13) **In progress. In the previous report we noted unexpected difficulties in infection of neurospheres with prion strains other than RML. We suspect that some of these difficulties are due to the tissue culture medium we have been using. A different source and type of medium has been identified and we are now testing whether our new culture conditions will allow infection with the 301V prion isolate, which was used in our in vivo studies. The new medium, NeuroCult NSC Basal Medium from StemCell Technologies, was specifically designed for CNS stem cell culture.**

*Task 3.* Validate putative prion-specific gene expression signatures as a blood test to identify prion-infected mice before clinical signs of disease appear. (Months 13-18)
**In progress. DEGs predicted by massively parallel signature sequencing to encode proteins solely expressed in the CNS and predicted to be secreted into blood are under analysis and differential expression of two of these proteins in the plasma of infected mice has been detected. These studies are continuing.**

*Task 4.* Establish neurosphere cultures from transgenic mice over-expressing human PrP (three lines) or bovine PrP (one line). (Months 1-4) **Completed. The cell lines are now in the BSL-3 laboratory of our collaborator, Dr. Stanley B. Prusiner, Director of the Institute for Neurodegenerative Diseases (IND), University of California, San Francisco (UCSF). Studies at UCSF also have been hampered by difficulties with Neurobasal medium and/or its supplements. Evaluation of NeuroCult NSC Basal Medium is being performed at UCSF in parallel with our evaluation in Montana.**

*Task 5.* Determine whether neurosphere lines from transgenic mice over-expressing human or bovine PrP can be infected with human and bovine prions. (Months 4-12) **In progress. Attempts to infect neurosphere lines with human or bovine will emphasize changes in culture conditions.**

*Task 6.* Establish methods for sensitive bioassay of prions in tissue culture using neurospheres from transgenic mice overexpressing mouse PrP. (Months 1-18) **In progress. A sensitive assay that recapitulates genetic susceptibility in vivo has been developed for RML prions. We are working to develop methods to allow detection of other mouse prion strains or prions from other species.**

**Task 1.** As reported previously, Dr. Inyoul Lee at the Institute for Systems Biology (ISB) performed a pilot study on two of our mouse CNS stem cells derived from two individual FVB Tg(MoPrP-A)B4053 mice (designated Tg1 and Tg2). Quantitation of transcript abundances in these samples was successful. Networks based on DEGs found in prion-infected mice have been constructed and will be integrated with those from our neurosphere studies.

**Task 2.** As reported previously, CNS neurosphere lines have been produced from FVB/NCr, C57BL/6J, B6.I, FVB.129-*Prnp*$^{tm1Zrch}$ (PrP-null) mice and those expressing PrP have been infected with the Rocky Mountain Lab (RML) prion strain to compare DEGs in neurospheres, which don't show obvious cytopathic effects prior to differentiation, and DEGs in prion-infected mice.

To complete our comparison, we need to be able to infect neurospheres with the mouse adapted BSE strain 301V (and other strains) as well as with RML. This has proven difficult. We now believe that at least some of this difficulty was due to an unknown change in the medium and/or growth factors. Beginning in August, our neurosphere cultures started growing more slowly, showed increased cell death, and RML prion-infected lines lost $PrP^{Sc}$. These difficulties with neruosphere growth occurred simultaneously in Dr. Prusiner's lab at UCSF and at Montana State University in Bozeman with neurosphere cultures established earlier in the summer at MRI. The complete medium is complex and each component was evaluated individually: Neurobasal medium, N2 growth supplement, EGF, bFGF, and LIF. The possibility of mycoplasma contamination was also tested. Neurobasal and N2 supplement have a single U.S. supplier, but alternative sources for the growth factors were tried. Results pointed to a change in either the Neurobasal Medium or N2 growth supplement, though nothing

could be identified by the manufacturer. We are now testing NeuroCult NSC Basal Medium with supplements and growing the fetal brain-derived neurospheres in the presence only of a single growth factor, EGF. The results so far are very encouraging and we should have results from prion-infection under these new growth conditions within a few weeks.

**Task 3.** Although difficulties involving ability to infect with prion strains in addition to RML and the technical problems outlined above have slowed our DEG analyses of neurosphere cultures, we are progressing in exploiting our existing data toward developing a preclinical blood signature to identify prion-infected individuals. Of the 934 DEGs common to our five standard host strain-prion strain combinations, ~100 are predicted as brain restricted transcripts by computational searches against transcriptome libraries of MPSS (Massively Parallel Signature Sequencing) on 40 tissues. Approximately 50 of these proteins are predicted to be secreted, thus holding promise as blood markers, and 8 of these showed differential gene expression beginning halfway through the incubation period—well before the onset of clinical signs. Expression patterns for these eight genes in each host-prion combination and in *Prnp* null mice are illustrated in Figure 1.

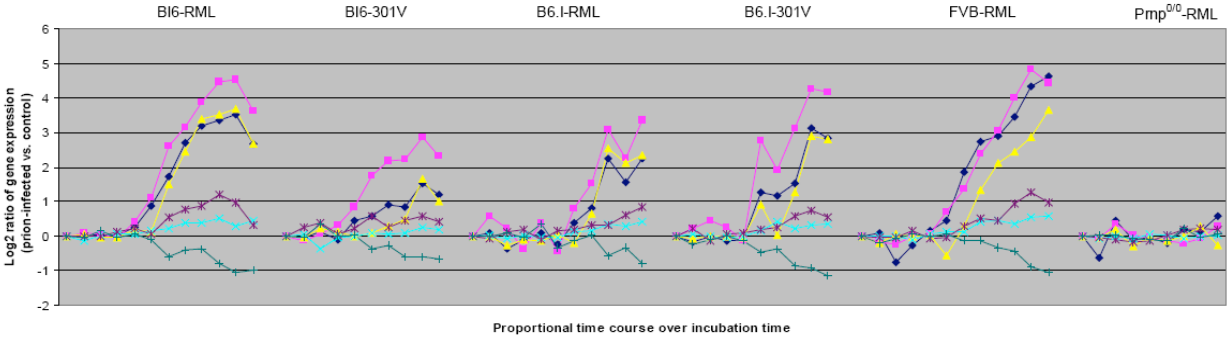
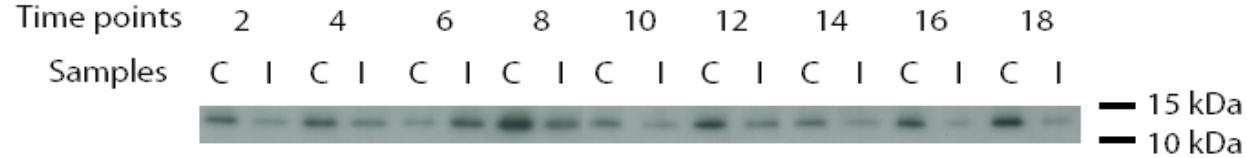



Figure 1. Eight genes predicted to be secreted proteins show differential expression. The incubation periods are normalized to emphasize the similarities among the groups. Note the constant levels of expression in *Prnp* null mice that cannot be infected with prions.

Preliminary Western blot results by Dr. Hyuntae Yoo at the Institute for Systems Biology show a decrease in plasma concentration for two of these brain-specific proteins. These preliminary results are illustrated in Figure 2.

**Protein 1**




**Protein 2**


Figure 2. Western blot analysis of two brain-specific proteins in plasma of 301V prion-infected B6.I mice and control mice over time, which is indicated in weeks after prion inoculation. C = plasma from mice injected with normal brain homogenate, I = plasma from mice injected with 301V brain homogenate.

**Task 4.** Completed. The required neurosphere cell lines have been established.

**Task 5**. Due to the difficulties described in Task 2 there is no progress since the previous submission to report at this time.

**Task 6.** Due to the difficulties described in Task 2 there is no progress since the previous submission to report at this time.

## KEY RESEARCH ACCOMPLISHMENTS

Produced stem cell neurosphere lines from the mouse strains used for dynamic analysis of gene expression following prion infection in vivo.

Identified DEGs specific to RML prion infection of Tg(MoPrP-A)4053 neurospheres.

Developed a data integration strategy for identification and validation of prion disease-specific biomarkers.

Developed a streamlined procedure for intracellular proteome analysis of cells from CNS stem cell containing neurosphere cultures.

Identified differentially expressed proteins in the plasma of prion-infected mice.

Isolated neurosphere lines from transgenic mice expressing human or bovine PrP.

## REPORTABLE OUTCOMES, October 2006- March 2007.

Two invited seminars describing this work were presented. Each was entitled “Host Responses to Prion Infection: A systems approach to prion replication and pathogenesis”.

1. Mt. Sinai School of Medicine, New York, NY, January 2007
2. PrP Canada 2007 Research Conference, Calgary, Alberta, February 2007

## CONCLUSIONS

Neurosphere cultures offer a powerful tool for dissecting prion disease. The project is being extended due to the technical difficulties described in this report. Progress is being made to correct the problems and we anticipate successful completion of the project as originally described before October 2007.